Characterization of New Illudanes, Illudins F, G, and H from the Basidiomycete *Omphalotus nidiformis*

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This investigation reports the isolation and characterization of three new illudane-type sesquiterpenes (**1**−**3**) from *Omphalotus nidiformis*, a Basidiomycete native to Australia and usually found in eucalypt forests in the eastern region of the country. These compounds are closely related to metabolites of North American and European *Omphalotus* species.

Omphalotus nidiformis (Berk.) Miller (formerly *Pleurotus nidiformis* and *Pleurotus lampas*) is generally found growing saprophytically on fallen *Eucalyptus* trunks or around the base of live standing *Eucalyptus* species where it has been reported to cause a white heart rot. Although this species is thought to be found only in Australia, it is part of a group of related luminescent fungi found worldwide and belongs to the genus *Omphalotus* Fayod within the family Paxillaceae. Despite its attractive appearance, *O. nidiformis* is mildly but not fatally poisonous, causing vomiting after a few hours.[1]

The first report of naturally occurring illudins was made in 1950 by Anchel and colleagues, who isolated crystalline illudin M and illudin S from *Clitocybe illudens* (now *Omphalotus olearius*) and demonstrated antibiotic activity of these compounds against *Staphylococcus aureus* and *Mycobacterium smegma*.[2] A range of other illudin sesquiterpenes is now known.[3,4] The toxicity of *Omphalotus* species has been well documented, and poisonings, producing symptoms of nausea, vomiting, double vision, and hallucinations, have been reported.[5,6,7] In Japan several fatalities resulting from the ingestion of an *Omphalotus* species have been recorded.[8]

Illudins and structurally related compounds have been targeted as potential anticancer agents, as they exhibit toxicity toward most tumor cells after prolonged exposure. Selective toxicity has been observed toward human myelocytic leukaemia and epidermoid, lung, ovarian, and breast carcinoma cells of various species of origin.[9]

Three new sesquiterpenes, belonging to the illudin family have been isolated and characterized during the course of a survey of metabolites produced by liquid shake cultures of the Australian Basidiomycete, *O. nidiformis*.[10] This paper gives the full experimental evidence that led to the structural and stereochemical assignments of these products.

Results and Discussion

Illudin F (**1**) was isolated as an oil. The molecular formula was $C_{15}H_{20}O_4$, determined by HREIMS. The UV spectrum of **1** in MeOH exhibited a maximum at 286 nm (ϵ 4600), which is consistent with the presence of an α,β-unsaturated ketone in conjugation with an exocyclic methylene group, according to the Woodward rules (calcd λ_{max} 290 nm).[11]

Full structural assignment of **1** was made by interpretation of 2D NMR spectra, including homonuclear COSY and NOESY experiments and inverse HSQC and HMBC experiments. The 1D ^{1}H NMR spectrum showed a simple set of resonances, characteristic of an illudane-type sesquiterpene. Four highfield multiplets were observed, each equivalent to one proton and characteristic of a cyclopropane moiety. Three tertiary methyl groups were observed between δ 1.07 and 1.33. Four broadened singlets (δ 4.59, 4.79, 5.28, and 5.77) were also observed, characteristic of methine protons of secondary alcohols and olefinic resonances. The HSQC experiment indicated the presence of only eight carbon atoms with protons attached. One olefinic carbon resonance (δ 115.5) correlated with the protons at δ 5.28 and 5.77, indicating the presence of an exocyclic methylene group. Two carbon resonances (δ 79.3 and 83.4) correlated with the proton resonances at δ 4.59 and 4.79, respectively, and are two secondary alcohols. A carbon resonance at δ 13.8 correlated with the cyclopropane protons at δ 0.20 and 1.14. A ^{13}C resonance at δ 5.0 exhibited correlation to the remaining cyclopropane proton resonances at δ 0.97 and 1.04.

The structure of **1** was finally deduced from the detailed assignment of the HMBC NMR spectrum and a through-space NOESY experiment. The HMBC NMR spectrum exhibited two methyl resonances in the ^{1}H dimension, at δ 1.07 and 1.18, with strong correlation to a quaternary carbon signal at δ 47.4 and the two secondary alcohols at δ 79.3 and 83.4 in the ^{13}C dimension. Bearing in mind that the illudane (or a closely related) skeleton was expected, the structure **1** was proposed.

Structure **1** was confirmed by several other long-range correlations observed in the HMBC and NOESY experiments. In the HMBC NMR spectrum, the cyclopropane protons correlated with carbons of the six-membered ring. In particular, proton H-11$_\alpha$ (δ 0.20) showed correlations to C-3, C-12, and C-2, and H-11$_\beta$ showed correlations to C-3, C-12, and C-2. The H-12$_\alpha$ and H-12$_\beta$ both correlated with C-2, C-3, C-4, and C-11. The olefinic proton H-13$_a$ showed correlations to C-3, C-11, and C-5, while the H-13$_b$ proton showed correlations to C-3, C-4, and C-5. The proton attached to C-8 showed correlations to C-15 as well as to C-9 and C-5. These assignments were confirmed, and the relative stereochemistry was assigned by interpretation of the NOESY NMR spectrum. The proton at δ 4.59 (H-6) showed a strong NOE cross-peak to the ge*m*-methyl group of C-15, as well as a strong cross-peak to the resonance at δ 5.77 (H-13$_a$), which was correlated to H-13$_b$ (δ 5.28).

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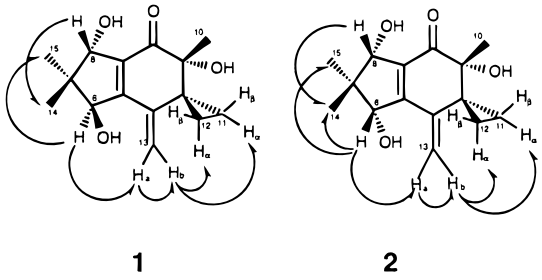
H-13$_b$, in turn, showed correlations to the cyclopropane resonances at δ 0.20 (H-11$_\alpha$) and 1.04 (H-12$_\alpha$).

The relative orientation of the C-10 methyl group and the C-10 hydroxyl group with respect to the cyclopropane ring is also apparent from the NOESY experiment. The C-10 methyl protons (δ 1.33) show an NOE cross-peak to the proton at δ 0.97, which is attached to C-12. Therefore, the remaining unassigned cyclopropane proton at δ 1.14 must be attached to C-11, the relatively lowfield chemical shift being consistent with its location cis to the hydroxyl substituent. The relative orientations of the hydroxyl groups at C-6 and C-8 are somewhat less definite. The proton at C-6 shows a strong cross-peak to the methyl at δ 1.18 (H$_3$-15), while the proton attached to C-8 exhibits a strong cross-peak to the methyl group at δ 1.07 (H$_3$-14). Therefore, the hydroxyl groups at C-6 and C-8 have different relative stereochemistry.

Illudin G (**2**), an isomer of **1**, was obtained as an oil. The molecular formula was established as $C_{15}H_{20}O_4$ by HREIMS. The 1D ^{1}H NMR spectrum of **2** is very similar to the NMR spectrum obtained for **1**, exhibiting a simple set of resonances characteristic of an illudane-type sesquiterpene. The main differences in the ^{1}H NMR spectra of **1** and **2** are the chemical shifts of H-6 and the protons of the C-14 methyl group. Also, there are minor differences in the ^{1}H NMR resonances of H-8, H-12$_\alpha$ and the protons of the C-15 methyl group. These differences in proton chemical shifts suggest that the hydroxyl group at C-6 is inverted in configuration and the H-6 proton in **2** shows more long-range coupling than the corresponding proton in **1**. There is the possibility of long-range W-coupling across the five-membered ring of **2**. The structure of illudin G was fully confirmed by interpretation of COSY, HSQC, HMBC, and NOESY NMR experiments.

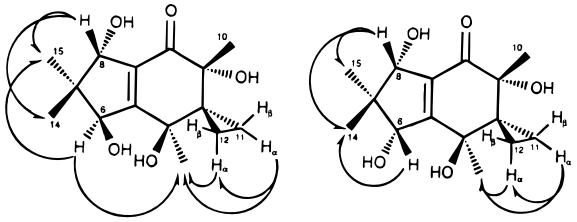
Compounds **1** and **2** possess very similar chromophores (expected UV λ_{max} 290 nm, ϵ 10 000−15 000). Illudin F (**1**) (found UV λ_{max} 286 nm, ϵ 4600) and illudin G (**2**) (found UV λ_{max} 284 nm, ϵ 2900) exhibited a bathochromic shift of 4 and 6 nm, respectively, compared to the calculated value. This may be attributed to increased steric hindrance, which is known to cause a bathochromic shift of 5−8 nm.[11] The large decrease in extinction coefficient compared to the expected value is probably due to the loss of coplanarity of the carbonyl and double-bond π electron systems. These values support the stereochemical assignments of **1** and **2** that were deduced from the NMR experiments. The changes in chemical shifts of the cyclopropane ring protons must indicate a change in conformation of the six-membered ring, and the distortion caused by the high degree of substitution around the ketone. It is not surprising that changing the stereochemistry at C-6, that is, inverting the hydroxyl group, leads to different λ_{max} and extinction coefficients in the UV spectra of **1** and **2**.

Illudin H (**3**), an isomer of illudin B (**4**), was isolated as an oil. The molecular formula was established as $C_{15}H_{22}O_5$ by HREIMS. The UV spectrum of **3** in MeOH, exhibits a maximum at 250 nm (ϵ 7800), which is consistent with the presence of an α,β-unsaturated ketone, according to the Woodward rules (calcd λ_{max} 249 nm)[11] and the same value as observed for **4**. The ^{1}H NMR spectrum of **3** was very similar to that observed for **4**.[3] The only significant differences in the ^{1}H NMR spectrum are in the chemical shifts of H-6$_\beta$ (0.55 ppm difference) and the smaller difference in shifts of H-8$_\beta$, H-11$_\alpha$, and H-12$_\alpha$ (all less than 0.1 ppm difference). This suggests that **3** is an isomer of **4**, at position 6. A corresponding change in proton chemical shifts of the methyl groups of positions 14 and 15 would be expected for such an isomer, but were not observed. Hence, to determine the structure of **3**, full assignments of HMBC, HSQC, and NOESY NMR experiments were performed on **3** and **4**.



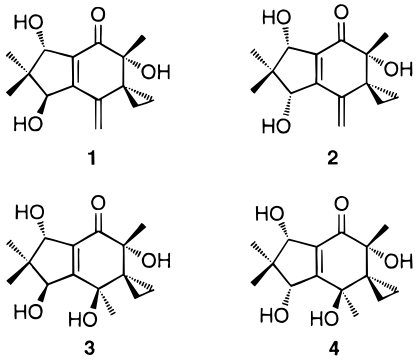
1 **2**

Figure 1. Some NOESY connectivities for illudin F (**1**) and illudin G (**2**).



3 **4**

Figure 2. Some NOESY connectivities for illudin H (**3**) and illudin B (**4**).



1 **2** **3** **4**

Minor differences in chemical shifts of **4** were observed compared to literature data for this compound.[3] However, these differences are probably due only to solvent effects. The relative stereochemistry determined for **4** is in agreement with the literature assignment. More significant differences were observed in the ^{13}C resonances of **3**, compared to **4**. These are most pronounced for the resonances of the C-14 (approximately 4.5 ppm difference) and C-15 (approximately 4.0 ppm difference) methyl groups. These results are expected for isomers at position 6 due to the shielding effect of the hydroxyl group attached to C-6. The probable relative stereochemistry and assignment of H-11$_\alpha$ and H-11$_\beta$ as well as H-12$_\alpha$ and H-12$_\beta$ were established by interpretation of the NOESY experiment. These results are shown diagrammatically in Figure 2.

Metabolites **1**−**3** were isolated from liquid shake cultures of *O. nidiformis* and are not present in significant amounts in extracts of the fruiting bodies, which contain lludin M and illudin S as the major metabolites. This may be because these liquid shake cultures were harvested early during secondary metabolite production before many oxidation reactions occurred and significant amounts of illudin M and S had not yet formed.[10]

Experimental Section

General Experimental Procedures. NMR spectra were acquired in $CDCl_3$ or CD_3COCD_3 solutions using a Bruker series DMX-600 spectrometer [600.13 MHz (^{1}H), 150.92 MHz (^{13}C)] and an inverse TXI probe. HSQC experiments employed coherence selection using shaped gradients[12] (gradient ratio: 40:10:40:−10). Typically, the spectral width in F2 was 2980 Hz, and 21 020 Hz in F1. In all, 256 data points were accumulated in the F1 dimension, and 2048 data points were used in the F2 dimension. Relaxation delays of 2.4 s duration were employed between pulses. The 2D matrixes were zero filled (1 K × 2 K) and processed with phase-shifted sine-squared functions in both dimensions.

A standard Bruker pulse program, supplied with the spectrometer, was employed for HMBC experiments with F2 spectral widths of 2900 Hz and F1 spectral widths of 32 000 Hz. Coherence selection using shaped gradients was employed with a gradient ratio of 50:30:40, and 4.7 s relaxation delays between pulses. Altogether, 256 data points were accumulated in F1, and 1024 data points were used in F2. After zero filling (1K × 4 K), the matrixes were processed with phase-shifted sine-squared functions in the *F*1 dimension and phase-shifted sine functions in the F2 dimension. NOESY spectra were acquired using a standard Bruker pulse program. Experiments had a spectral width of 3600 Hz in both dimensions and relaxation delays of 4.5 s between pulses. Altogether, 1024 data points were accumulated in F1, and 2048 data points were accumulated in F2, and the 2D matrixes were processed with phase-shifted sine functions in both dimensions.

Extraction and Isolation. *O. nidiformis* (B. J. Rees, culture no. UNSW 057) was maintained on malt extract agar, containing 20 g each of malt extract and agar per liter of water; pH was adjusted to 6.5, prior to sterilization. Seed cultures were prepared by inoculation of 300 mL of liquid malt-extract medium in 1-L baffled flasks with mycelia from 2-week-old plate cultures. These cultures were incubated at 23 °C with orbital shaking (100 rpm) in the dark. After 10 days of growth, cultures were homogenized in a Waring-type blender for two bursts of 1-s duration. The resulting suspension was used as a 10% inoculum for shake cultures (10 × 300 mL) in 1-L baffled flasks. Mycelium was collected by filtration using a nylon mesh membrane. The filtrate (2.4 L) was acidified to pH 2.5 using 0.1 M HCl, and secondary metabolites were extracted with EtOAc (2 × 0.5 vol). The extracts were combined and evaporated to dryness under reduced pressure at 35 °C using a rotary evaporator.

The crude extract (1.25 g) was dissolved in 1 mL of EtOAc and applied to a column of silica 60 H (8 × 3.5 cm i.d.), packed dry under suction, and equilibrated in hexane. Eight 100-mL fractions were eluted from the column in the following solvents: (a) hexane; (b) EtOAc−hexane (1:19 v/v); (c) EtOAc−hexane (3:17 v/v); (d) EtOAc−hexane (1:3 v/v); (e) EtOAc−hexane (2:3 v/v); (f) EtOAc−hexane (3:2 v/v); (g) EtOAc; (h) MeOH.

HPLC was performed using an Altex model 420 system controller programmer, Altex model 110A pumps, and a Spectra Physics Spectra 100 variable wavelength detector set at 254 nm. Fraction 6 obtained from vacuum−liquid chromatography contained **1** and **2**. Preliminary HPLC fractionation was achieved using a Waters Prep Nova-Pak HR C_{18} cartridge (100 × 25 mm i.d.) in conjunction with a Waters radial compression module. A flow rate of 5 mL/min was employed. Isocratic elution was performed using CH_3CN-H_2O (2:3 v/v) as solvent. The following five fractions were collected: 6.1 (t_R 8.0 min), 6.2 (t_R 9.0 min), 6.3 (t_R 12.6 min), 6.4 (t_R 15.4 min), and 6.5 (t_R 16.0 min). Each fraction was then applied to an Activon GoldPak 5μ ODS column (250 × 4.6 mm i.d.) with a flow rate of 1 mL/min. Further purification of illudin F (**1**) and illudin G (**2**) from fraction 6.2 was achieved by isocratic elution from HPLC using CH_3CN-H_2O (3:7 v/v). Illudin H (**3**) and illudin B (**4**) were isolated from vacuum−liquid chromatography fraction 7. Three major fractions were obtained from preparative reversed-phase HPLC using CH_3CN-H_2O (1:1 v/v) as eluent: 7.1 (t_R 20.6 min), 7.2 (t_R 28.0 min), and 7.3 (t_R 30.0 min). Illudin H was isolated from fraction 7.1 using a 5-μ ODS column (250 × 4.6 mm i.d.) and CH_3CN-H_2O (1:4 v/v) as eluent. Illudin B was isolated from fraction 7.3 by isocratic elution from HPLC using CH_3CN-H_2O (3:7 v/v).

Mass measurement was performed using a VG Autospec time-of-flight electrospray mass spectrometer. The samples were dissolved in MeOH and the mass measurements carried out with electrospray ionization. Infrared absorption spectra of illudins F, G, and H were acquired using an ATI Mattson Genis Series FT−IR instrument. The samples were dissolved in CH_2Cl_2, and five scans were accumulated between 400 cm^{-1} and 4000 cm^{-1}.

Illudin F (1): oil; UV (MeOH) λ_{max} (ϵ) 286 nm (4600); IR (CH_2Cl_2) ν_{max} 1669 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.20 (1H, m, H-12$_\alpha$), 0.96 (1H, m, H-11$_\alpha$), 1.04 (1H, m, H-12$_\beta$), 1.11 (3H, s, H-14), 1.11 (1H, m, H-11$_\beta$), 1.18 (3H, s, H-15), 1.33 (3H, s, H-10), 4.59 (1H,d, J = 6.4 Hz, H-6), 4.79 (1H, br s, H-8), 5.28 (1H, s, H-13a), 5.77 (1H, s, H-13b); ^{13}C NMR ($CDCl_3$) 4.8 (C-11), 13.6 (C-12), 16.8 (C-15), 25.3 (C-10), 26.0 (C-14), 30.5 (C-3), 46.0 (C-7), 75.4 (C-4), 78.6 (C-8), 83.4 (C-6), 115.3 (C-13), 136.3 (C-9), 142.9 (C-4), 160.2 (C-5), 202.1 (C-1); HREIMS *m*/*z* 264.1337 (calcd for $C_{15}H_{20}O_4$ 264.1362).

Illudin G (2): oil; UV (MeOH) λ_{max} (ϵ) 284 nm (2900); IR (CH_2Cl_2) ν_{max} 1675 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.20 (1H, m, H-11$_\alpha$), 0.97 (1H, m, H-12$_\alpha$), 1.06 (1H,m, H-12$_\beta$), 1.11 (3H, s, H-14), 1.14 (1H, m, H-11$_\beta$), 1.18 (3H, s, H-15), 1.33 (3H, s, H-10), 4.58 (1H, m, H-6), 4.79 (1H, s, H-8), 5.28 (1H, H-13b), 5.77 (1H, H-13a); ^{13}C NMR ($CDCl_3$) 4.8 (C-12), 13.6 (C-11), 16.8 (C-15), 25.3 (C-10), 26.0 (C-14), 30.5 (C-3), 46.0 (C-7), 75.4 (C-2), 78.6 (C-8), 83.4 (C-6), 115.3 (C-13), 136.3 (C-9), 142.9 (C-4), 160.2 (C-5), 202.1 (C-1); HREIMS *m*/*z* 264.1345 (calcd for $C_{15}H_{20}O_4$ 264.1362).

Illudin H (3): oil; UV (MeOH) λ_{max} (ϵ) 250 nm (7800); IR (CH_2Cl_2) ν_{max} 1670 cm^{-1}; ^{1}H NMR (CD_3COCD_3) δ 0.50 (1H, m, H-12$_\alpha$), 0.65 (1H, m, H-11a), 0.72 (1H, m, H-11b), 0.84 (1H, m, H-12$_\beta$), 0.97 (3H, s, H-14), 1.11 (3H, s, H-15), 1.25 (3H, s, H-13), 1.54 (3H, s, H-10), 4.37 (1H, s, H− 8), 4.39 (1H, s, H-6); ^{13}C NMR (CD_3COCD_3) δ 3.8 (C-11), 6.8 (C-12), 15.9 (C-14), 24.1 (C-13), 25.3 (C-15), 26.4 (C-10), 34.0 (C-3), 49.1 (C-7), 71.7 (C-4), 76.2 (C-2), 79.2 (C-8), 82.0 (C-6), 135.7 (C-9), 161.4 (C-5), 202.2 (C-1); HREIMS *m*/*z* 282.0525 (calcd for $C_{15}H_{22}O_5$ 282.1467).

Illudin B (4): oil; UV (MeOH) λ_{max} (ϵ) 250 nm (7800); IR (CH_2Cl_2) ν_{max} 1670 cm^{-1}; ^{1}H NMR (CD_3COCD_3) δ 0.48 (1H, m, H-12$_\beta$), 0.60 (1H, m, H-11$_\alpha$), 0.69 (1H, m, H-11$_\beta$), 0.86 (1H, m, H-12$_\alpha$), 0.94 (3H, s, H-14), 1.16 (3H, s, H-15), 1.28 (3H, s, H-13), 1.45 (3H, s, H-10), 4.29 (1H, d, H-8), 4.94 (1H, d, H-6); ^{13}C NMR (CD_3COCD_3) δ 3.5 (C-11), 6.8 (C-12), 20.4 (C-14), 21.3 (C-15), 24.9 (C-13), 27.7 (C-10), 30.0 (C-3), 47.8 (C-7), 71.3 (C-4), 74.9 (C-2), 78.7 (C-8), 82.7 (C-6), 134.7 (C-9), 163.9 (C-5), 201.3 (C-1).

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